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Overexpression of pitx3 upregulates expression of BDNF and GDNF in SH-SY5Y cells and primary ventral mesencephalic cultures

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Abstract **The transcription factor Pitx3 plays an important role in the development of midbrain to promote the growth and differentiation of dopamine neurons. The present study has demonstrated that overexpression of Pitx3 in SH-SY5Y cells and primary ventral mesencephalic (VM) cultures significantly increased the mRNA levels of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), and remarkably elevated the protein levels of these two neurotrophic factors. Our data provide the first evidence that pitx3-expressing cells are able to upregulate the expression of BDNF and GDNF. Therefore, Pitx3 might be a good target for the treatment of Parkinson's disease.**

Keywords: Pitx3; Brain-derived neurotrophic factor; Glial cell line-derived neurotrophic factor; SH-SY5Y cells; Ventral mesencephalon cultures

1. Introduction

Pitx3 is a homeodomain transcription factor and its expression in brain is highly restricted in the dopamine (DA) neurons of substantia nigra (SN) and ventral tegmental area (VTA) [1,2]. It has been reported that Pitx3-deficient aphakia mice failed to develop nigrostriatal pathway, showing a complete loss of DA neurons in the SN, while DA neurons in the VTA were less affected [3–5]. The DA levels in the dorsal striatum in the Pitx3-deficient mice were severely reduced by 84–93% that of wild-type mice [4,6,7]. Mesencephalic DA neurons and their immediate progenitors can be subdivided into two partially overlapping groups based on their temporal expression profiles of tyrosine hydroxylase (TH) and Pitx3: the ventrolateral mesencephalic cells express Pitx3 prior to TH, whilst the dorsomedial midbrain DA neurons express TH ahead of Pitx3 [8]. It is still unknown why the selective loss of DA neurons occurs in Pitx3-deficient mice. We hypothesize that Pitx3 may regulate the expression of genes which are necessary for DA neuron survival. Here we report that exogenous expression of Pitx3 in SH-SY5Y cells and primary ventral mesencephalic (VM) cultures regulates the expression of brain derived neurotrophic factor (BDNF) and glial derived neurotrophic factor (GDNF).

2. Materials and methods

2.1. Plasmid construction

PCR products for Pitx3 were amplified by RT-PCR from mouse midbrain total mRNA using the RT-PCR system (Promega, USA) with the following primers: forward 5′ ACA GCC ACC ACC CGG AGT 3′ reverse 5′ GCA CCC CTT TCA GAC CCT 3′, and cloned into pUCm T-vector (Sangon, China). Then Pitx3 cDNA was subcloned into pcDNA3.1(+) (Invitrogen, USA) and sequenced to confirm identity with the Pitx3 mRNA sequence in NCBI database (NM_008852).

2.2. Stable transfection and isolation of Pitx3-expressing SH-SY5Y cell clones

SH-SY5Y cells were grown on poly-D-lysine (Sigma, USA) precoated dishes in Dulbecco's modified Eagle's medium (DMEM, GIBCO-Invitrogen, USA) supplemented with 10% fetal bovine serum (heat-inactivated, GIBCO-Invitrogen). SH-SY5Y cells were transfected with Pitx3-pcDNA3.1 or pcDNA3.1 using lipofectamine 2000 (Invitrogen). Stably transfected cells were selected with 600 μg/ml Neomycin (DUCHEFA, Holand). Neomycin-resistant clones were expanded and total proteins from each clone were collected in RIPA lysis buffer. Clones expressing pitx3 were identified by Western-blot analysis using anti-pitx3 antibody (Zymed, USA).

2.3. Primary mesencephalic cultures and transfection

Primary embryonic rat (embryonic day 14, E14; Experimental Animal Center of Shanghai) VM cells were isolated and cultured according to the method described previously [9]. VM cells were transfected with Pitx3-pcDNA3.1 or pcDNA3.1 using lipofectamine2000 after six days cultured in the serum-free medium, consisting of DMEM/Ham's F12 (1:1) with the addition of 2% B27 supplement (Sigma). Protein or RNA was extracted from VM cells after three days of cultures.

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Abbreviations: AADC, amino acid decarboxylase; BDNF, brain-derived neurotrophic factor; DA, dopamine; DAT, dopamine transporter; GDNF, glial cell line-derived neurotrophic factor; RT-PCR, reverse transcriptase-polymerase chain reaction; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VM, ventral mesencephalon; VTA, ventral tegmental area

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Animal care and procedures were performed in accordance with the Laboratory Animal Care Guidelines approved by Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences.

2.4. Immunocytochemistry

Cells were fixed in 4% formaldehyde for 30 min, rinsed with PBS, and then incubated with blocking buffer (PBS, 5% normal horse serum; Vector Labs, USA) for 30 min. Cells were then incubated overnight at 4 °C with rabbit anti-pitx3 antibody (Zymed). After additional rinsing in PBS, cells were incubated with TRITC-conjugated anti-rabbit antibody or biotinylated anti-rabbit IgG (Vectastain Elite ABC kit, Vector Labs, USA) for 2 h at RT. After rinsing for 3 × 10 min in PBS, cells for immuno-fluorescent staining were incubated with Hoechst for 5 min and examined under the fluorescent microscope; cells for diaminobenzidine (DAB) staining were incubated with avidin-biotinylated-peroxidase complex for 1 h (Vectastain Elite ABC kit, Vector Labs). Immunoreactivity was visualized by DAB substrate (Vector Labs), and examined under the light microscope.

2.5. Semiquantitative RT-PCR analysis

Total RNA from cultured cells was prepared using SV Total RNA Isolation System (containing DNase I, Promega). For RT-PCR analysis, 2 μg of RNA was transcribed into cDNA with the Reverse Transcription System (Promega) and oligo (dT) primers in 20 μl volume. The cDNA was then analyzed in a PCR assay using the primers as given in Table 1. PCRs were carried out in 1× reaction buffer, 0.2 μM of each primer, 1 μl cDNA and 2.5 units of *Taq* DNA polymerase (Promega).

2.6. Western-blot analysis

Cells were extracted in ice-cold RIPA lysis buffer. Lysates were then centrifuged for 10 min at 14000 × *g* at 4 °C, 40 μg of protein from each supernatant was loaded onto 12% SDS–PAGE and transferred to polyvinylidene difluoride, blocked for 1 h in 5% non-fat milk, and incubated with primary antibodies [anti-pitx3 (Zymed), anti-β-actin (Sigma), anti-BDNF (Promega) and anti-GDNF (Promega)]. Incubations took place overnight at 4 °C. After washing 3 × 10 min in TBST, samples were incubated with peroxidase-conjugated secondary antibody for 2 h, then washed in TBST and developed with Super Signal® West Dura Extended Duration Substrate (Pierce Biotechnology, USA). The identified proteins were quantified by measuring optical densities of the bands.

2.7. Measurement of BDNF and GDNF by ELISA

The medium from treated and untreated cell cultures was concentrated up to four fold using centrifugal filter devices (Amicon® Ultra-4 Ultracel-10k, Millipore). The protein concentration of the samples was adjusted to the same levels before 100 μL of each sample was applied into each well (Maxisorpa 96-well plate, Nunc) for the immunoassay. The protein concentration of BDNF and GDNF was measured using BDNF or GDNF $E^{®}_{max}$ immunoAssay System (Promega) and the procedures performed per manufacturers' instruction.

3. Results

3.1. Exogenous expression of Pitx3 in nucleus

We constructed the expression vector PcDNA3.1-Pitx3 to generate Pitx3-overexpressing SH-SY5Y cell clones. Two clones (P1 and P4) were found to express Pitx3 (upper panel of Fig. 1). Immunochemical analysis showed that exogenous expression of Pitx3 was present in the nucleus (mid panel, Fig. 1C) and pitx3 was not detected in two controls (SHC2, an empty vector-transfected clone, Fig. 1A and naïve SH-SY5Y, Fig. 1B). Using immuno-fluorescent staining with Hoechst we found that Pitx3 was only located in SH-SY5Y cell nuclei (lower panel, Fig. 1F).

3.2. Overexpression of Pitx3 upregulates mRNA expression of BDNF and GDNF in SH-SY5Y cells

We hypothesized that Pitx3 may regulate genes that are important for the development and survival of DA neurons, such as neurotrophic factors and their receptors. So we analyzed gene expressions of several neurotrophic factors (i.e.,

Table 1
Primer

Gene	Forward primer Reverse primer	Length of product (bp)	T_m (°C)	Cycles
Pitx3	5′GAGCACAGTGACTCGGAGAAGG3′ 5′AAGGCGAACGGGAAGGTC3′	413	60	26
GAPDH	5′CCATGTTCGTCATGGGTGTGAACCA3′ 5′GCCAGTAGAGGCAGGGATGATGTTC3′	251	58	25
BDNF	5′AGCGTGAATGGGCCCAAGGCA3′ 5′TGTGACCGTCCCGCCCGACA3′	363	60	28
GDNF	5′CTGGGCTATGAAACCAAGGA3′ 5′GACAGGTCATCATCAAAGGC3′	173	54	31
TrkB	5′ATGAGTATGGGAAGGATG3′ 5′ATGCCAAACTTGGAGTGT3′	298	58	28
Nurr1	5′GAGTCTGATCAGTGCCCTCGTC3′ 5′CTTCCACTCTCTTGGGTTCCTTG3′	437	54	28
TH	5′GCAGTTCTCGCAGGACATTG3′ 5′TGGATGCGTGAGGCATAGC3′	321	54	30
AADC	5′TTACTCATCCGATCAGGCACAC3′ 5′GGCAGAACAGTCAAAATTCACC3′	385	57	28
GFRα1	5′AGACCATCGTGCCTGTGTGCT3′ 5′GGTCATGACTGTGCCAATAAG3′	215	56	35
NGF	5′CCACCGCCACAGACATCAAG3′ 5′TCGGCAGGTCAGGCTCTTCT3′	295	60	32
NGFR	5′GCCTGAAGTTGGAGTGAGTGT3′ 5′AGGTGATAGGAGGGTTGCTG3′	401	57	30
BDNF (Rat)	5′AGCGTGAATGGGCCCAGGGCA3′ 5′TGTGACCGTCCCACCGGACA3′	369	60	30
GDNF (Rat)	5′TTGGGCTACGAAACCAAGGA3′ 5′GACAGGTCGTCGTCGAAGGC3′	173	54	35
GAPDH (Rat)	5′CCATGTTTGTGATGGGTGTGAACCA3′ 5′GCCAGTGGATGCAGGGATGATGTTC3′	251	58	25

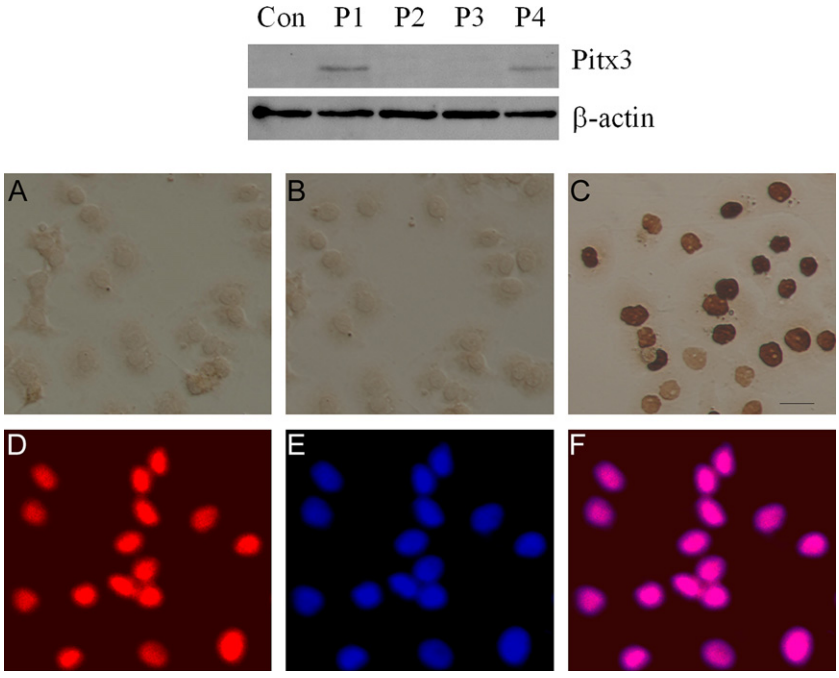

Fig. 1. Isolation of SH-SY5Y cell clones expressing Pitx3. Upper panel: Western blot analysis showed that Pitx3 protein was detected in samples of P1 and P4 clones, whereas none was seen in naïve SH-SY5Y cells (Con lane). Mid panel: immunochemistry analysis of Pitx3. (A) SHC2 cells, an empty vector-transfected clone; (B) naïve SH-SY5Y cells; (C) Pitx3-expressing SH-SY5Y cells. Lower panel: Immuno-fluorescent staining of Pitx3. (D) Pitx3-expressing SH-SY5Y cells; (E) Hoechst staining of nuclear; (F) overlay of (D) and (E). Scale bar, 10 μm.

BDNF, GDNF, NGF, and FGF) and their corresponding receptors (i.e., TrkB, GFRα1, NGFR, and FGFR), in addition of various DA markers (i.e., TH, Nurr1, AADC and DAT), in the Pitx3-expressing SH-SY5Y cells.

We found that mRNA levels of BDNF and GDNF were 1.7- and 1.3-fold higher in Pitx3-expressing SH-SY5Y cells than two controls cells (SHC2, an empty vector-transfected and naïve SH-SY5Y cells), respectively, while their corresponding receptors TrKb and GFRα1 were downregulated (Fig. 2). The expression of mRNA of NGF and its receptor was not obviously changed in Pitx3-expressing SH-SY5Y cells as compared to controls (Fig. 2). The expression of mRNA of FGF and its receptor FGFR was not detected in all samples after PCR amplification over 45 cycles (Fig. 2). While expressions of TH, Nurr1, DAT (data not shown), and AADC mRNAs were not visibly altered by Pitx3 transfection (Fig. 2).

3.3. Upregulation of BDNF and GDNF proteins by transgene expression of Pitx3

Consistent with the mRNA changes, the proteins of BDNF and GDNF were increased 10-, 5.9-fold, respectively, in Pitx3-expressing SH-SY5Y cells as compared with controls (Fig. 3). ELISA showed that the protein level of BDNF secreted by Pitx3-expressing SH-SY5Y cells was increased 4.6-fold over controls (SH-Pitx3, 12.3 ± 0.93 pg/ml, controls, 2.7 ± 0.24 pg/ml and 2.6 ± 0.22 pg/ml, $n = 3$, Fig. 3), and the protein level of GDNF secreted from Pitx3-expressing SH-SY5Y cells was increased 2.5-fold over controls (SH-Pitx3, 9.1 ± 0.69 pg/ml, controls, 3.7 ± 0.30 pg/ml and 3.6 ± 0.33 pg/ml, $n = 3$, Fig. 3).

3.4. Increased expression of BDNF and GDNF by Pitx3 transfection in VM cultures

Pitx3 transfection in primary VM cultures increased mRNA levels of BDNF and GDNF by 1.4-, 1.5-fold, and upregulated protein levels of BDNF and GDNF by 1.9-, 1.7-fold (Fig. 4 right), respectively, as compared with pcDNA3.1-transfected controls (Fig. 4 left).

4. Discussion

BDNF and GDNF are the two most important neurotrophic factors for the differentiation and survival of midbrain DA neurons [10–12]. In addition, both BDNF and GDNF can protect DA neurons against neurotoxins in vivo and in vitro [13–15]. Moreover, BDNF protein produced by midbrain DA neurons not only provides a neurotrophic effect on the DA neurons itself, but it is also transported in a retrograde manner to affect striatal neurons function [15–17]. GDNF expression was detectable in the ventral midbrain at embryonic day (E) 18 and postnatal day one and continued to present at substantia nigra pars compacta (SNc) and VTA neurons in adult rodents [18–20]. Moreover, expression of the GDNF receptor complex, GFRa1 and Ret, was detectable in SNc region by E10, before striatal innervation [21]. These findings support the notion that GDNF promotes DA neurons survival and differentiation in an autocrine and/or paracrine fashion in the developing and adult midbrain.

Our study demonstrated for the first time that Pitx3 can upregulate the expression of these two neurotrophic factors in SH-SY5Y cells as well as in primary VM cultures at both transcriptional and translational levels. In this study, we also found that TrkB and GFRα1, the neurotrophic factor receptors of BDNF and GDNF, respectively, were downregulated in the Pitx3-expressing SH-SY5Y cells, presumably through the negative feedback regulation responding to the dramatic increase of the ligands BDNF and GDNF. It is yet to determine in the future study the molecular mechanisms by which

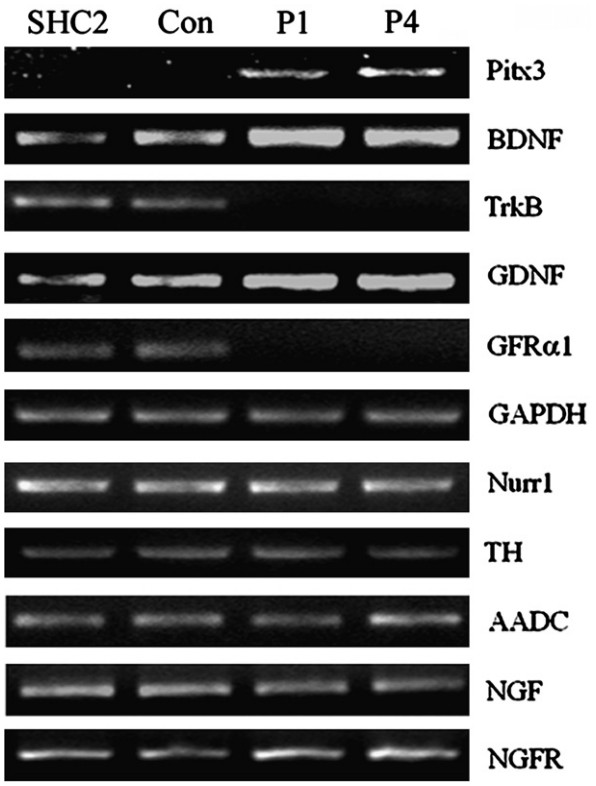

Fig. 2. Semiquantitative RT-PCR analysis of mRNA differences between Pitx3-expressing SH-SY5Y and controls (SHC2 and Con, naive SH-SY5Y). RNA samples were prepared from Pitx3-expressing clones P1 and P4. For comparisons, we used SHC2 cells (an empty vector-transfected clone) and naïve SH-SY5Y cells.

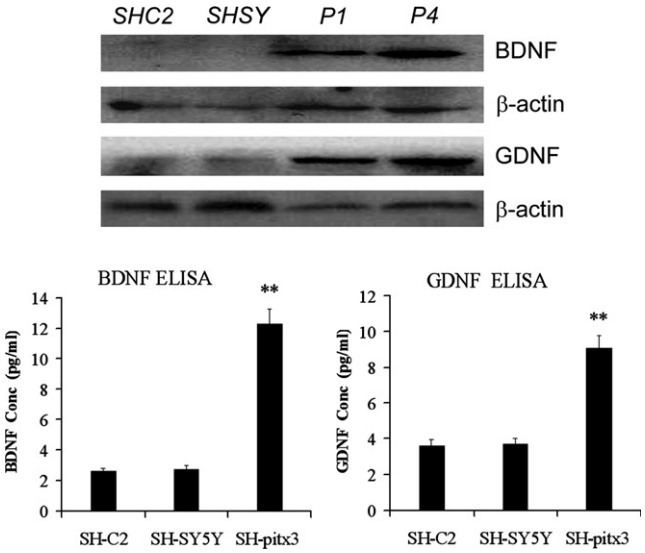

Fig. 3. Upregulation of protein levels of BDNF and GDNF in Pitx3-expressing SH-SY5Y. Upper panel: Tested by Western blot analysis, protein levels of BDNF and GDNF increased 10- and 5.9- fold in Pitx3-expressing SH-SY5Y cells, respectively, when compared with controls (SHC2 cells and Con, naïve SH-SY5Y cells). β-Actin served as an internal control. Lower panel: increase of BDNF and GDNF proteins secreted by Pitx3-expressing SH-SY5Y cells. The content of BDNF and GDNF released into the culture medium (pg/ml) was measured by ELISA. Data shown are means ± S.E.M. values from three independent experiments performed in triplicate. Data analysis were performed by using one-way ANOVA followed by post hoc S-N-K multiple comparisons with the SPSS 13.0 program (SPSS). A *P*-value of <0.05 was considered significant (**,$P < 0.01$).

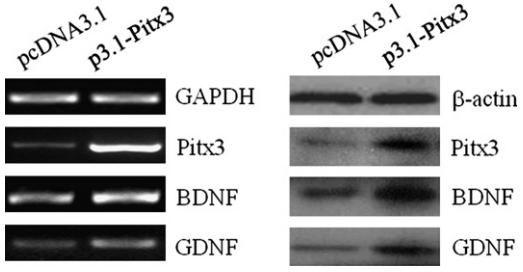

Fig. 4. Increased expression of BDNF and GDNF by Pitx3 transfection in primary VM cultures. Both mRNA levels (left) and protein levels (right) of BDNF and GDNF were increased in Pitx3-transfected VM cultures as compared with pcDNA3.1-transfected VM cultures.

Pitx3 regulates BDNF and GDNF expression. Furthermore, the findings in vitro may need further verification in Pitx3-over expressed transgenic mice and in Pitx3-deficient mice to determine whether BDNF and GDNF expression is altered by Pitx3 in vivo.

Using semiquantitative PCR analyses we showed that mRNA expression of general DA markers such as Nurr1, TH, AADC, and DAT were unchanged or only marginally affected in the Pitx3-expressing SH-SY5Y cells. These results were consistent with the report of Chung et al., who found overexpression Pitx3 did not alter expression general DA markers such as TH and DAT in final differentiation of ES cells [22]. It has been reported that Nurr1-null mice did not show TH-expression in E12.5 embryonic midbrain but did have Pitx3-expressing neurons in E11.5 embryonic midbrain [23]. Pitx3-deficient mice scarcely changed the TH expression pattern in E11.5 embryonic midbrain [5]. These results indicate that Pitx3 and Nurr1 independently control two aspects of specification of DA neurons in the development of the midbrain.

In conclusion, our study demonstrated that Pitx3 upregulated the mRNA and protein expressions of BDNF and GDNF in vitro, and elevated the release of BDNF and GDNF to the culture medium. Our findings shed new light on our understanding of the gene regulation and molecular pathway of Pitx3 acting on the survival of midbrain DA neurons, and suggest that Pitx3 might be a potential new drug target for the treatment of Parkinson's disease.

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